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Systems Pharmacology Analysis of an Estrogen-Modulating Polyherbal Formulation for Reversing Age- and Sex-Related Cognitive Decline

Mayank Roy Chowdhury*

Department of Biotechnology, National Institute of Technology, Andhra Pradesh, India

*Corresponding author: Mayank Roy Chowdhury, mayank.roy74@gmail.com

Abstract

Background: Alzheimer's disease (AD) exhibits pronounced sex bias, with estrogen decline emerging as a critical contributor to accelerated cognitive deterioration in aging females. Therapeutic strategies capable of restoring estrogen-mediated neuroprotection without systemic hormone replacement remain an unmet clinical need. Methods: This study employed an integrative systems pharmacology framework to evaluate the estrogen-modulating potential of a rational polyherbal phytoformulation comprising *Bacopa monnieri* (BM), *Hippophae rhamnoides* (HR), and *Dioscorea bulbifera* (DB). ADMET (Absorption, Distribution, Metabolism, Excretion and toxicity) filtered phytoconstituents underwent target prediction, protein-protein interaction topology analysis, Gene Ontology and KEGG enrichment, molecular docking, and molecular dynamics simulations, with mechanistic prioritization centered on estrogen receptor-1 (ESR1). Results: Network analysis identified ESR1 as the dominant hub-bottleneck regulator, functionally linked to steroidogenesis, lipid metabolism, and endocrine signaling. Enrichment profiling confirmed strong convergence toward estrogen receptor signaling, steroid hormone biosynthesis, ovarian steroidogenesis, and endocrine resistance pathways. Structure-based validation demonstrated stable occupation of the ESR1 ligand-binding pocket by four phytoconstituents, with emodin and β-sitosterol showing binding affinities comparable to or exceeding estradiol. Subsequent 25-ns molecular dynamics simulations revealed sustained structural stability and minimal residue-level fluctuations, supporting robust ESR1 engagement. Conclusion: Collectively, these findings define an ESR1-centered, estrogen-restorative neuroprotective mechanism underlying the investigated polyherbal formulation and highlight phytoestrogen-driven multi-target modulation as a promising complementary strategy for age- and sex-associated cognitive decline in AD. This work provides a mechanistically grounded foundation for advancing endocrine-modulating phytotherapeutics toward translational neuroprotection.

Keywords

Alzheimer's disease, Estrogen receptor-1, Systems pharmacology, Polyherbal phytoformulation, Neuroprotection

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory impairment, executive dysfunction, and extensive neuronal loss driven by amyloid-β accumulation, tau hyperphosphorylation, neuroinflammation, synaptic failure, and metabolic dysregulation [1]. Despite decades of intensive research, currently approved pharmacotherapies—including acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists—remain largely symptomatic, providing only modest and transient cognitive benefits without halting disease progression [2]. Even recently developed disease-modifying strategies demonstrate limited clinical efficacy and raise safety concerns, highlighting the urgent need for complementary, mechanism-oriented therapeutic approaches capable of addressing the multifactorial pathobiology of AD [3].

A critical yet under-addressed dimension of AD is its pronounced sex disparity. Women constitute nearly two-thirds of AD cases globally and frequently exhibit faster cognitive decline and greater disease severity than men—differences that cannot be explained solely by increased female longevity. Growing evidence implicates menopause-associated estrogen depletion, sex-specific immune and metabolic alterations, and dysregulated estrogen receptor signaling in enhancing susceptibility to neurodegeneration. Estrogen functions as a key neuroprotective regulator of synaptic plasticity, mitochondrial bioenergetics, neuroinflammatory balance, and amyloid-tau homeostasis; consequently, disruption of estrogen signaling represents a mechanistic bridge linking aging, sex, and cognitive decline [4,5].

Although hormone replacement therapy (HRT) has been explored to restore estrogenic neuroprotection, its clinical utility remains limited due to inconsistent cognitive outcomes and elevated risks of cardiovascular complications and hormone-sensitive malignancies [6]. These limitations underscore the importance of alternative strategies capable of selectively modulating estrogen-responsive neuroprotective pathways without systemic hormonal exposure [7]. However, currently available pharmacological interventions rarely incorporate sex-informed or endocrine-targeted neuroprotective mechanisms in a holistic manner [8].

Polyherbal formulations derived from traditional medicinal systems present a promising complementary strategy. Owing to their chemically diverse phytoconstituents, such formulations can exert synergistic effects across multiple molecular targets and signalling cascades implicated in AD, including neurotrophic regulation, anti-inflammatory activity, oxidative stress attenuation, and endocrine modulation [9]. When applied as adjuvant therapies, polyherbal interventions may enhance the efficacy of conventional treatments rather than replace them. Nevertheless, their rational translation into modern neurotherapeutics requires systematic, mechanism-based validation [10].

Systems pharmacology offers a powerful integrative framework for elucidating the multi-target actions of complex phytomedicines. By combining pharmacokinetic screening, blood-brain barrier (BBB) permeability (BBBp) assessment, target prediction, protein-protein interaction network analysis, pathway enrichment, and molecular docking and dynamics simulations, this approach enables deconvolution of polyherbal synergy and identification of biologically relevant estrogen-modulating mechanisms associated with cognitive aging [11,12].

In the present study, we investigate BHD, a rationally designed polyherbal phytoformulation composed of *Bacopa monnieri* (BM), *Hippophae rhamnoides* (HR), and *Dioscorea bulbifera* (DB). BM is widely recognized for cognitive-enhancing, antioxidant, anti-amyloid, and synaptic plasticity-promoting properties, with emerging links to estrogen-responsive neurotrophic signaling. HR exhibits neuroprotective, anti-inflammatory, and mitochondrial-supportive activities and activates PI3K-AKT and ERK pathways overlapping with estrogen-mediated neuronal survival. DB, rich in steroidal saponins and diosgenin precursors, demonstrates estrogen-modulatory, cholinergic, and anti-amyloid effects in experimental and network-pharmacology studies [13,14].

Collectively, these characteristics position BHD as a promising estrogen-modulating adjuvant capable of addressing age- and sex-associated cognitive decline at a systems level. Using an integrative systems pharmacology strategy, this study aims to predict the estrogen-modulating potential of BHD, delineate its multi-target neuroprotective mechanisms, and establish a mechanistic foundation for its supportive role alongside existing AD therapies.

2. Material and Methods

2.1 Small Molecule Screening

A comprehensive library of phytochemical constituents was compiled from rigorously curated literature sources and established phytochemical databases, including TCMSP (https://www.tcmsp-e.com/load_intro.php?id=43) [15] and IMPPAT 2.0 (https://cb.imsc.res.in/imppat/) [16], and subsequently utilized for downstream screening and analysis. Pharmacokinetic characterization of the collected compounds was conducted through ADME profiling using SwissADME (http://www.swissadme.ch/) [17] alongside Molsoft (https://molsoft.com/mprop/). To identify pharmacologically relevant candidates, compounds were filtered according to critical drug-discovery criteria, including oral bioavailability, drug-likeness, and BBBp. Only molecules satisfying the thresholds of OB ≥30%, DL ≥0.18, and BBBp ≥-0.3 were retained as putative active constituents. In addition, strict compliance with Lipinski's Rule of Five—encompassing molecular weight, calculated lipophilicity (CLogP), and hydrogen-bond donor and acceptor counts—was enforced to ensure drug-like physicochemical properties [18]. Following ADME-based prioritization, the shortlisted compounds underwent systematic *in silico* toxicity evaluation using the ProTox-III platform (https://tox.charite.de/protox3/index.php?site=home) [19]. This assessment comprehensively examined potential

toxicological liabilities, including hepatotoxicity, neurotoxicity, cardiotoxicity, cytotoxicity, and carcinogenicity, thereby enabling safety-informed selection of candidate phytochemicals for subsequent analyses.

2.2 Target Prediction and Disease Association

To investigate the potential molecular targets and disease associations of phytocompounds that successfully passed ADME and toxicity screening, a structured two-tier *in silico* workflow was implemented. Target prediction analysis was performed exclusively on the shortlisted compounds from earlier screening stages to ensure both pharmacokinetic suitability and safety relevance. Putative molecular targets were identified using BindingDB (https://www.bindingdb.org/rwd/bind/index.jsp) [20] and SwissTargetPrediction (http://www.swisstargetprediction.ch/) [21], employing the SMILES representations of each screened compound as input. BindingDB was specifically applied to detect protein targets exhibiting ≥85% structural similarity, after which the corresponding gene symbols were standardized through the UniProt database (https://www.uniprot.org/) [22]. Concurrently, SwissTargetPrediction generated a probability-ranked list (Top 25) of candidate protein targets for each compound based on a structure-activity relationship (SAR)-driven predictive framework. Redundant targets identified across both platforms were systematically removed to obtain a non-redundant consolidated target set.

Because modulation of Estrogen Receptor-1 (ESR1) is not expected for all screened phytochemicals, SwissTargetPrediction was further employed to evaluate the probabilistic likelihood of ESR1 interaction. For each compound, the ten highest-probability predicted targets were examined, and compounds in which ESR1 appeared within this top-ranked subset were classified as potential ESR1 modulators. Only these high-confidence ESR1-associated candidates were advanced for subsequent ESR1-focused mechanistic analyses.

2.3 Protein-Protein Interaction Mapping

To investigate the functional relationships among the previously identified overlapping gene set, a comprehensive PPI network was generated using the STRING database (https://string-db.org/) [23], which integrates both experimentally validated interactions and computationally predicted association data. The network analysis was restricted to first-order interaction partners and filtered using a medium confidence interaction threshold (score ≥0.7) to retain biologically meaningful and reliable connections. In order to improve structural interpretability and minimize analytical noise, redundant interaction edges as well as isolated nodes lacking connections (singletons) were systematically excluded from the final network topology. To further delineate functionally coherent substructures within the interaction landscape, cluster detection was performed using the Markov Cluster Algorithm (MCL). An inflation parameter of 3 was applied to enable identification of densely interconnected protein modules based on shared connectivity patterns, thereby facilitating recognition of biologically relevant functional groupings within the PPI network.

2.4 Hub-Bottleneck Identification

To further refine the identification of key regulatory genes within the PPI framework, a hub-bottleneck filtering strategy grounded in network topology was implemented. In the constructed interaction network, nodes correspond to genes or proteins, whereas edges represent their functional or physical associations. Fundamental to network-based analysis are topological descriptors such as degree–defined as the number of direct interactions linked to a node–and betweenness centrality, which quantifies the frequency with which a node lies along the shortest communication paths between other nodes. Nodes exhibiting high degree values are considered network hubs due to their extensive connectivity, while those with elevated betweenness centrality function as bottlenecks, playing essential roles in mediating information flow across the network. To systematically determine hub and bottleneck candidates, the average degree (A.D.) and corresponding standard deviation (SD) of the network were first calculated. Nodes surpassing the threshold of A.D. + 2 × SD were designated as hub genes. Concurrently, the top 5% of nodes ranked by betweenness centrality were classified as bottleneck genes. Genes satisfying both hub and bottleneck criteria were ultimately defined as hub-bottleneck genes, following the methodological framework described by [24]. These critical regulatory nodes, together with their first-degree neighboring interactors, were subsequently extracted and visualized using Cytoscape, enabling detailed interpretation of their structural importance and potential functional influence within the broader interaction network.

2.5 Gene Ontology and Pathway Enrichment Analysis

To obtain functional insight into the biological roles of the identified hub-bottleneck genes, a comprehensive enrichment analysis was conducted using the ShinyGO v0.77 platform (http://bioinformatics.sdstate.edu/go/) [25], with Homo sapiens specified as the reference organism. Stringent statistical cutoffs of $p < 0.05$ and false discovery rate (FDR) < 0.05 were applied to ensure robustness and minimize false-positive associations, and enriched terms were prioritized according to fold enrichment values. The analysis generated the top ten significantly enriched Gene Ontology (GO) terms across the three principal functional domains–Biological Process, Cellular Component, and Molecular Function–thereby delineating the dominant functional themes associated with the input gene set. To complement GO-based functional characterization, KEGG pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes database (https://www.genome.jp/kegg/) [26]. Pathways meeting the significance threshold ($p < 0.05$) were further interrogated using the Pathview tool [27], which enabled mapping of gene expression relevance and identification of overlapping core regulatory genes shared among the top ten enriched signaling pathways. These core genes were visually emphasized in red, facilitating intuitive recognition of their central regulatory positions within

complex signaling cascades. To further contextualize the systems-level relationships between plant-derived bioactive compounds, hub-bottleneck genes, and enriched pathways, an integrated interaction network was constructed using Cytoscape. This graphical framework illustrates potential pathway cross-talk and functional connectivity among enriched biological modules, thereby providing mechanistic insight into how coordinated gene-pathway interactions may contribute to the molecular pathophysiology of AD.

2.6 Molecular Docking Analysis

To clarify the molecular basis through which selected bioactive phytocompounds may regulate hub-bottleneck genes associated with AD, a focused molecular docking-based structural analysis was conducted. Based on prior network topology, enrichment profiling, and ESR1-centered pathway prioritization, the docking investigation was restricted to a single high-confidence therapeutic target–ESR1 thereby ensuring strong biological relevance while maintaining computational efficiency. Correspondingly, only the four ESR1-associated phytocompounds identified during target prediction (β-sitosterol, stigmasterol, emodin, and diosgenin) were advanced for structural interaction analysis. The three-dimensional crystal structure of the estrogen receptor-α ligand-binding domain in complex with estradiol (PDB ID: 1A52) was retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/) [28]. Structural coordinates of the selected phytocompounds were obtained from the PubChem database. To improve structural stability and docking reliability, the receptor protein underwent energy minimization using the YASARA minimization server (http://www.yasara.org/minimizationserver.htm) [29]. Subsequently, protein preparation and structural optimization were performed in PyMOL (https://pymol.org/), including removal of co-crystallized ligands, solvent molecules, and other non-essential heteroatoms, followed by saving the refined receptor structure in PDB format for downstream docking. Molecular docking simulations were executed using AutoDock Vina 1.1.2 (https://vina.scripps.edu/) [30], with the graphical interface of UCSF Chimera (https://www.rbvi.ucsf.edu/chimera/) [31] employed to facilitate grid generation, docking execution, and pose visualization. This ESR1-focused docking strategy, grounded in prior systems pharmacology and enrichment evidence, enabled mechanistically coherent evaluation of estrogen-modulating interactions between prioritized phytocompounds and the receptor's ligand-binding domain, thereby providing structural validation for the proposed estrogen-centered neuroprotective mechanism of the polyherbal formulation.

2.7 Molecular Dynamic Simulations

To further evaluate the structural stability and dynamic behavior of the docked ESR1-phytocompound complexes, molecular dynamics simulations were performed using the Desmond simulation package from Schrödinger LLC [32] for a total simulation time of 25 ns. Prior to simulation, the ESR1 protein structure underwent optimization and energy minimization using the Protein Preparation Wizard in Maestro to eliminate steric clashes and ensure appropriate geometric configuration [33]. Simulation systems were generated using the System Builder module, employing the SPC (Simple Point Charge) solvent model within an orthorhombic simulation box and parameterized with the OPLS_2005 force field [34,35]. Counter-ions were introduced to achieve electrical neutrality, and 0.15 M NaCl was added to reproduce physiological ionic strength. Following system construction, equilibration was carried out under constant temperature (300 K) and pressure (1 bar) conditions using sequential NVT ensemble equilibration with a V-rescale thermostat and NPT ensemble equilibration with a Parrinello-Rahman barostat, each for 100 ps. Production trajectories were subsequently recorded, with coordinates saved at 100-ps intervals for downstream analysis. The stability and conformational integrity of each ESR1-ligand complex throughout the simulation period were assessed using root mean square deviation (RMSD) analysis, enabling time-resolved evaluation of protein-ligand structural fluctuations and overall dynamic stability.

3. Results

3.1 Identification of Active Phytochemicals and Toxicity Network Profiling

Systematic pharmacokinetic screening of the curated phytochemical library yielded a subset of bioactive compounds that satisfied predefined drug-likeness and brain-penetration criteria. Application of oral bioavailability, drug-likeness, and BBBp thresholds, together with strict compliance with Lipinski's Rule of Five, enabled prioritization of pharmacologically relevant candidates suitable for neurotherapeutic investigation. The finalized list of screened active phytoconstituents, along with their physicochemical and ADME characteristics, is summarized in Table 1.

Subsequent *in silico* toxicity assessment using the ProTox-III platform indicated that the majority of shortlisted compounds exhibited low predicted toxic liability across key safety endpoints, including hepatotoxic, neurotoxic, cardiotoxic, cytotoxic, and carcinogenic risks. Only a limited number of molecules demonstrated moderate toxicity predictions, suggesting an overall favorable safety profile for the selected phytochemical set. To further elucidate compound-toxicity relationships at a systems level, an interaction network integrating phytochemicals with their predicted toxicological endpoints was constructed. This toxicity association network, illustrated in Figure 1, highlights the distribution of safety liabilities among the screened compounds and supports the identification of phytoconstituents with optimal pharmacological relevance and minimal adverse risk. Collectively, these findings establish a safety-filtered pool of neuroactive phytochemicals suitable for downstream target prediction and mechanistic network pharmacology analyses.

Table 1. ADME properties and drug-likeness parameters of selected phytocompounds meeting screening criteria and Lipinski's Rule of Five.

S.No	PC ID	Phytocompound Name	PubChem ID	Plants	MW.	BBBp	OB%	DL	RO5	Ref.
1	PC16	Diosgenin	99474	DB	414.60	0.27	80.88	0.81	Y	[36]
2	PC26	Sapogenin	4483642	DB	486.70 YES	1.83	56.00	0.20	Y	[37]
3	PC2	Betulinic acid	64971	BM	456.78	0.22	55.38	0.78	Y	Screened
4	PC24	Bafoudiosbulbin F	102479350	DB	404.4	0.43	55.00	0.29	Y	Screened
5	PC23	Bafoudiosbulbin A	101410701	DB	402.4	0.25	55.00	0.44	Y	Screened
6	PC21	Dioscorine	442635	DB	221.29	0.48	55.00	0.77	Y	[37]
7	PC22	Jarnol	5318869	DB	314.29	-0.22	50.83	0.29	Y	Screened
8	PC4	Stigmasterol	5280794	BM, DB	412.77	1.00	43.83	0.76	Y	[38]
9	PC18	Diosbulbin B	9974762	DB	344.39	0.46	43.01	0.70	Y	Screened
10	PC28	Diosbulbin A	177106	DB	376.44	-0.28	39.52	0.65	Y	[37]
11	PC15	Oleanolic acid	10494	HR	456.78	0.07	29.02	0.76	Y	Screened
12	PC30	Emodin	3220	DB	270.25	-0.26	30.12	0.24	Y	Screened
13	PC3	Beta-Sitosterol	222284	BM, HR, DB	414.69	1.00	51.00	0.25	Y	Screened

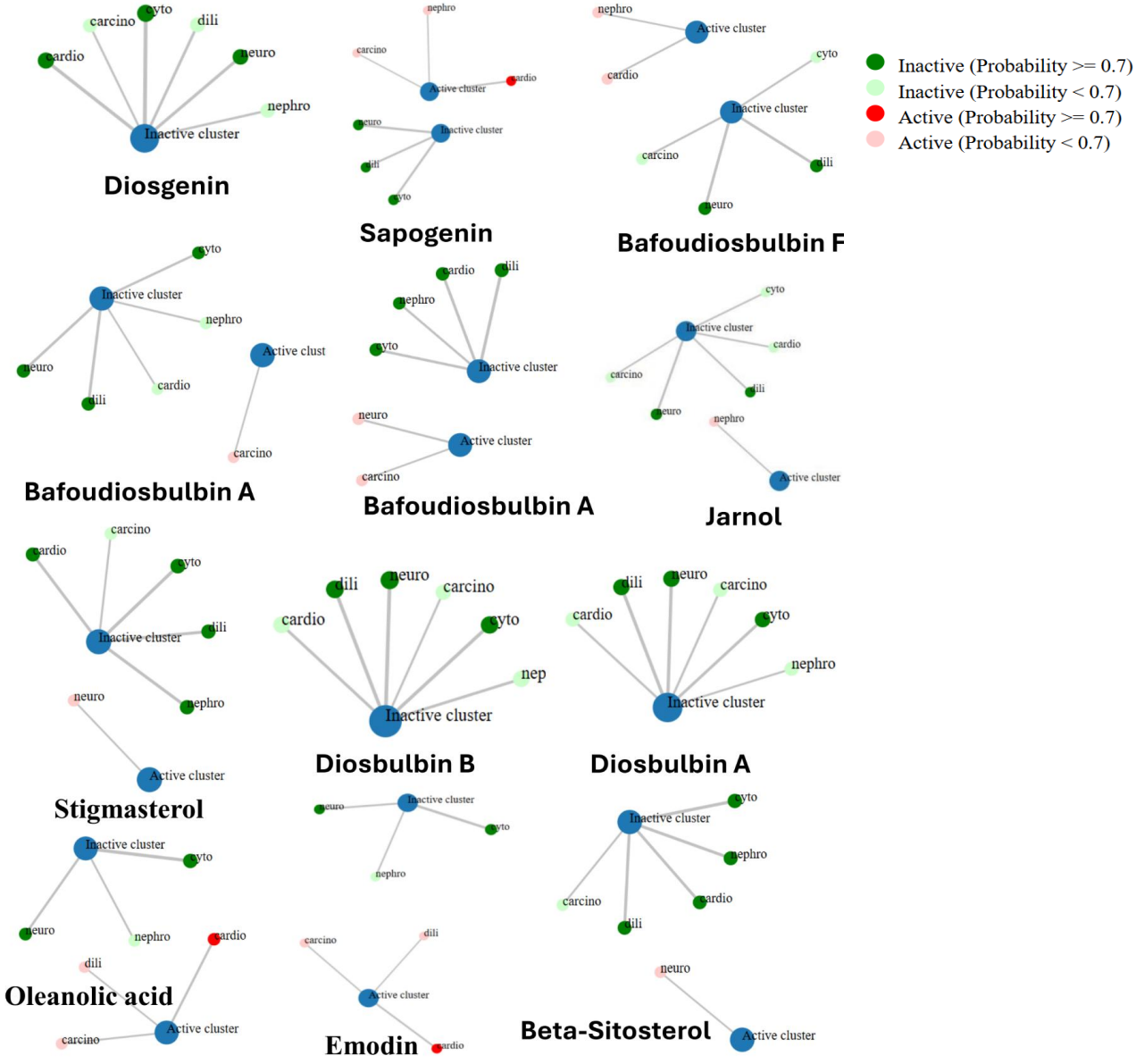

Figure 1. *In silico* toxicity clustering of ADME-screened phytocompounds based on ProTox-III predictions. Green nodes indicate inactive toxicity predictions (dark green: probability ≥0.7; light green: probability <0.7), while red nodes denote active toxicity predictions (dark red: probability ≥0.7; light red: probability <0.7). Toxicity endpoints assessed include hepatotoxicity, neurotoxicity, cardiotoxicity, cytotoxicity, nephrotoxicity, and carcinogenicity.

3.2 Target Prediction and Identification of Esr1-Associated Phytocompounds

Implementation of the two-tiered target prediction framework on phytochemicals that satisfied ADME and toxicity screening criteria generated a consolidated, non-redundant repertoire of putative molecular targets derived from BindingDB and SwissTargetPrediction analyses. Integration of structural similarity-based matching with probability-driven structure-activity relationship modeling ensured the identification of biologically plausible protein targets associated with the screened compounds. Considering the pivotal involvement of ESR1 in estrogen-mediated neuroprotective signaling, a focused probabilistic evaluation was undertaken to determine which phytochemicals were most likely to interact with ESR1. Assessment of the top ten highest-probability predicted targets for each compound revealed that only four phytoconstituents—β-sitosterol, stigmasterol, emodin, and diosgenin—contained ESR1 within their top-ranked target list, consistent with the predefined methodological criteria. The complete compound-wise predicted target datasets are now provided as Supplementary Table 1 (diosgenin), Supplementary Table 2 (emodin), Supplementary Table 3 (stigmasterol), and Supplementary Table 4 (β-sitosterol), ensuring full data transparency, traceability, and reproducibility of the target prediction workflow.

These high-confidence ESR1-associated compounds were therefore prioritized for subsequent mechanistic analyses. To maintain pathway specificity and minimize background network complexity, only the molecular targets corresponding to these four ESR1-linked phytochemicals were employed for the construction of the PPI network (33 unique) targets for 4 compounds and downstream systems-level investigations. The detailed list of ESR1-associated compounds and their predicted targets is provided in Supplementary Table 5, forming the basis for estrogen-focused network pharmacology interpretation in the following sections.

3.3 PPI Network Topology and Functional Module Organization

To elucidate the systems-level relationships among the ESR1-associated target genes, a PPI network was constructed using STRING and subsequently refined according to the predefined confidence and clustering criteria. The finalized interaction network comprised 32 nodes and 60 edges, markedly exceeding the expected 15 edges, thereby indicating a significantly enriched interaction landscape (PPI enrichment $p < 1.0 \times 10^{-16}$). Topological analysis further revealed an average node degree of 3.75 and an average local clustering coefficient of 0.414, supporting the presence of functionally coordinated protein connectivity rather than random associations. These parameters collectively suggest that the ESR1-linked phytocompound targets participate in biologically coherent signalling modules relevant to estrogen-mediated neuroprotection and cognitive regulation. Application of MCL-based modular detection enabled identification of densely interconnected protein clusters, highlighting potential functional sub-networks that may cooperatively regulate neuroinflammatory balance, synaptic signalling, and hormone-responsive survival pathways. The overall PPI interaction architecture derived from ESR1-associated targets is illustrated in Figure 2, providing a systems-level framework for interpreting downstream pathway enrichment and mechanistic analyses.

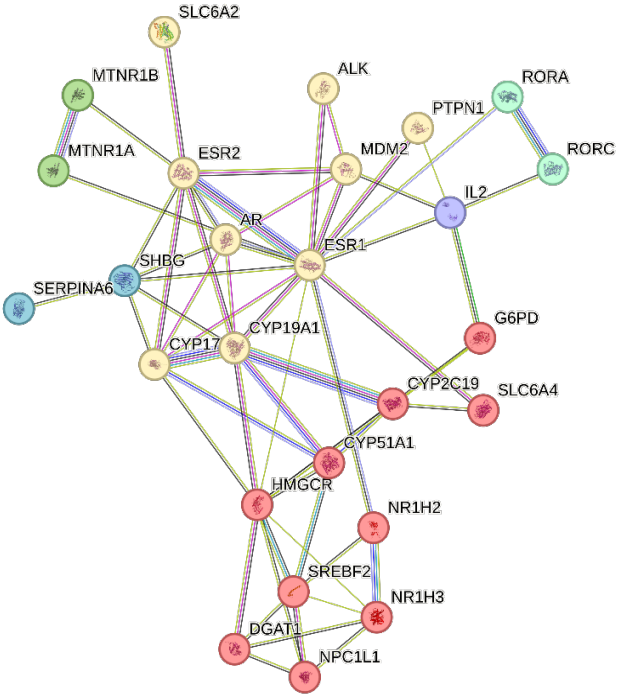

Figure 2. PPI network of ESR1-associated target genes. The interaction map was generated using the STRING database with a medium confidence threshold (≥0.4) and restricted to first-order interactions. The final network consists of 32 nodes and 60 edges, significantly exceeding the expected number of interactions (15 edges; PPI enrichment $p < 1.0 \times 10^{-16}$). Network topology demonstrates an average node degree of 3.75 and an average clustering coefficient of 0.414, indicating non-random, functionally coordinated protein connectivity. MCL clustering (inflation = 3) highlights densely connected functional modules potentially involved in estrogen-mediated neuroprotective signaling and cognitive regulation.

3.4 Hub-Bottleneck Gene Prioritization and Network Centrality Analysis

Comprehensive topological interrogation of the ESR1-associated PPI network using the predefined hub-bottleneck filtering framework enabled the systematic identification of structurally central and functionally influential regulatory genes. Application of the hub threshold (A.D. + 2 × SD) highlighted nodes with markedly elevated connectivity, while concurrent selection of the top 5% betweenness centrality values (corresponding to a degree cutoff ≥6) ensured retention of genes exerting critical control over information flow within the interaction architecture. Integration of these criteria yielded eight hub-bottleneck genes: ESR1, HMGCR, CYP19A1, ESR2, CYP17A1, SREBF2, AR, and SHBG. Among them, ESR1 exhibited the highest degree (14) and maximal betweenness centrality (0.4902), clearly positioning it as the dominant regulatory core of the estrogen-responsive interaction network. The pronounced centrality of ESR1, together with strong contributions from HMGCR and CYP19A1, indicates functional convergence between steroidogenesis, cholesterol metabolism, and estrogen signalling pathways, reinforcing the mechanistic relevance of estrogen modulation in age- and sex-associated cognitive decline. Visualization of the hub-bottleneck subnetwork further illustrates this hierarchical organization, where node size and colour intensity scale with degree centrality, enabling intuitive recognition of dominant regulators. In this representation, ESR1 appears as the most prominent and highly connected node, underscoring its pivotal systems-level influence. The hub-bottleneck interaction landscape is depicted in Figure 3, providing structural support for prioritizing ESR1-centered signalling in subsequent pathway enrichment and mechanistic interpretation. The quantitative topological parameters supporting these observations are summarized in Table 2.

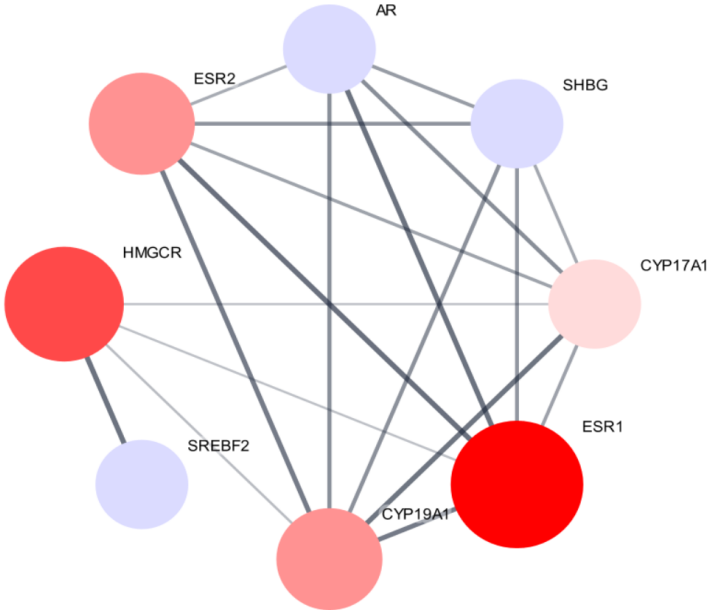

Figure 3. Hub-bottleneck interaction network of ESR1-associated regulatory genes. Nodes represent prioritized hub-bottleneck genes derived from PPI topological filtering (A.D. + 2 × SD hub threshold and top 5% betweenness bottleneck criterion). Node size and color intensity are proportional to degree centrality, highlighting hierarchical regulatory influence within the estrogen-responsive network. ESR1 emerges as the most highly connected and visually dominant node, indicating its central systems-level role linking steroidogenesis, cholesterol metabolism, and hormone-mediated neuroprotective signaling relevant to cognitive decline.

Table 2. Hub-bottleneck genes identified from ESR1-associated PPI topology.

Gene	Degree	Betweenness Centrality	Hub (A.D. + 2 × SD)	Top 5% Bottleneck
ESR1	14	0.490206	Yes	Yes
HMGCR	10	0.253004	Yes	Yes
CYP19A1	8	0.084206	Yes	Yes
ESR2	8	0.142119	Yes	Yes
CYP17A1	7	0.052179	Yes	Yes
SREBF2	6	0.014722	Yes	Yes
AR	6	0.005952	Yes	Yes
SHBG	6	0.080000	Yes	Yes

3.5 Gene Ontology and Pathway Enrichment Analysis

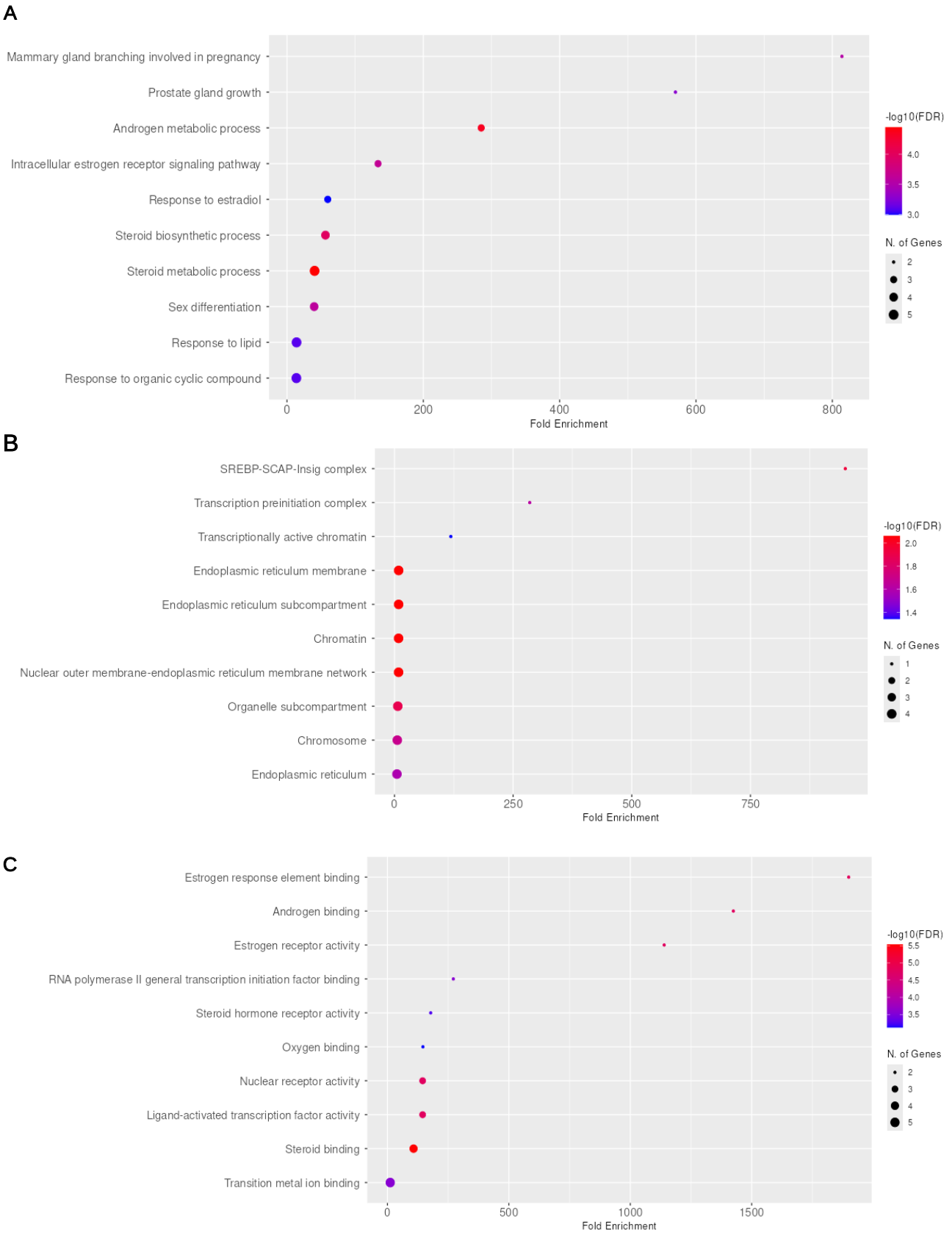

Figure 4. Gene Ontology (GO) enrichment of hub-bottleneck genes. (A) Biological Process: enrichment in steroid hormone regulation, intracellular estrogen receptor signaling, androgen metabolism, steroid biosynthesis/metabolism, sex differentiation, and estradiol response. (B) Cellular Component: localization to endoplasmic reticulum-associated membranes, chromatin, transcription pre-initiation complexes, nuclear membrane-ER network, and the SREBP-SCAP-Insig complex. (C) Molecular Function: overrepresentation of estrogen receptor activity, steroid hormone receptor activity, androgen binding, ligand-activated transcription factor activity, estrogen response element binding, and nuclear receptor activity. Dot size indicates gene count, color represents -log10(FDR), and the x-axis denotes fold enrichment.

To elucidate the biological relevance of the prioritized hub-bottleneck genes, comprehensive Gene Ontology (GO) and KEGG pathway enrichment analyses were performed. GO enrichment revealed strong functional convergence toward steroid hormone regulation, estrogen responsiveness, and endocrine-associated developmental processes. Within the Biological Process category, the most significantly enriched terms included intracellular estrogen receptor signaling, androgen metabolic process, steroid biosynthetic and metabolic processes, sex differentiation, and response to estradiol, collectively highlighting hormone-dependent regulatory mechanisms (Figure 4A & Supplementary Table 5). In the Cellular Component domain, enriched terms predominantly localized to endoplasmic reticulum-associated membranes, chromatin, transcriptional pre-initiation complexes, and the SREBP-SCAP-Insig regulatory complex, indicating that the identified genes function within lipid-regulatory, transcriptional, and steroidogenic subcellular compartments (Figure 4B & Supplementary Table 6). Similarly, Molecular Function enrichment demonstrated strong overrepresentation of estrogen receptor activity, steroid hormone receptor activity, androgen binding, ligand-activated transcription factor activity, and estrogen response element binding, confirming that the hub-bottleneck gene set is functionally centred on nuclear hormone receptor signalling (Figure 4C & Supplementary Table 7).

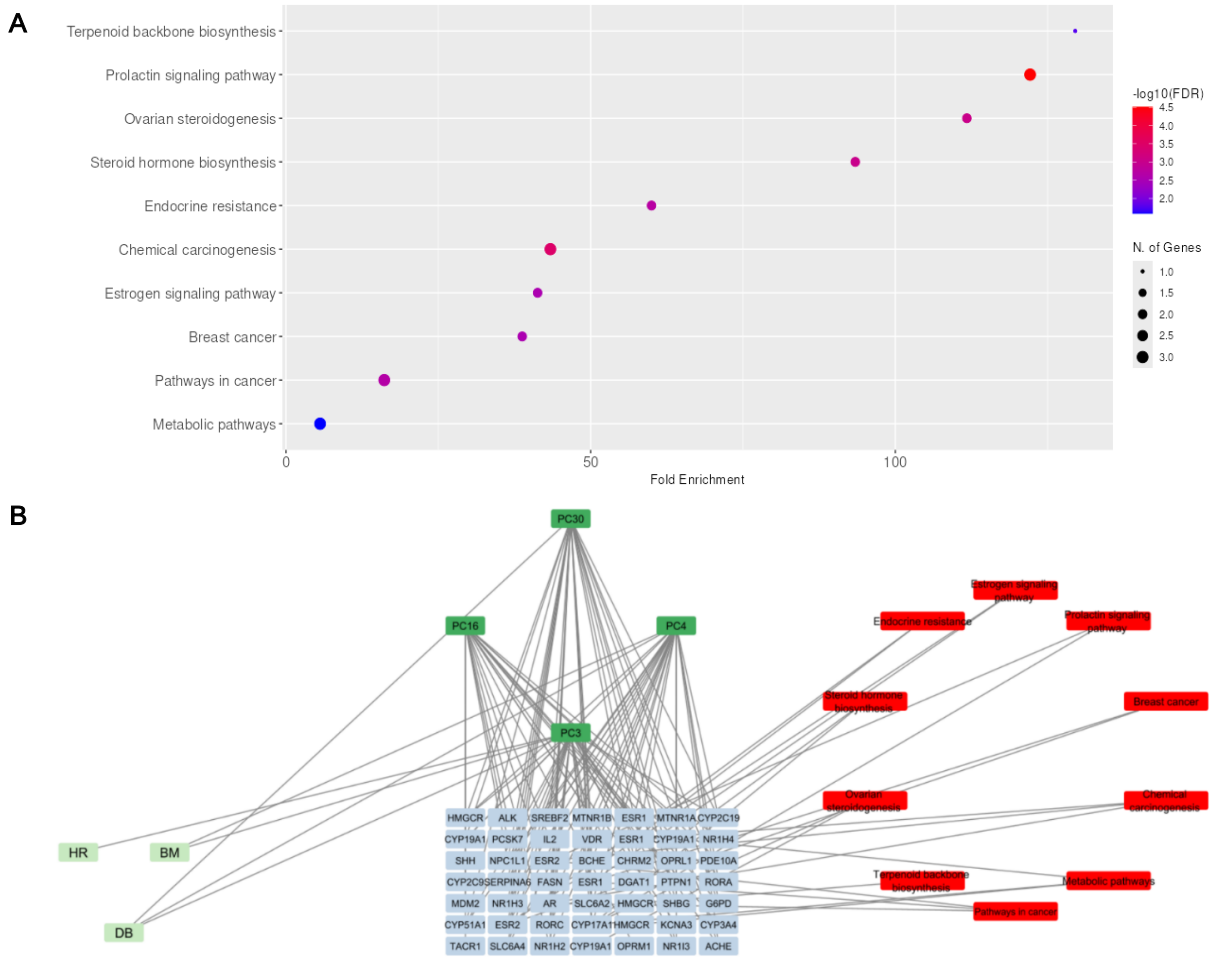

Figure 5. KEGG pathway enrichment analysis of hub-bottleneck genes. (A) Top enriched pathways include steroid hormone biosynthesis, ovarian steroidogenesis, prolactin signaling, endocrine resistance, estrogen signaling, metabolic pathways, and cancer-related pathways, highlighting convergence toward endocrine regulation and estrogen-mediated signaling. (B) Integrated plant-bioactive compound-target-pathway interaction network. The network illustrates relationships among polyherbal plant sources, their bioactive compounds, hub-bottleneck target genes, and enriched KEGG pathways, revealing multi-target connectivity, pathway cross-talk, and coordinated estrogen-centered regulatory mechanisms underlying the proposed therapeutic action. Dot size represents gene count, color indicates -log10(FDR), and the x-axis denotes fold enrichment.

Consistent with GO findings, KEGG pathway enrichment identified ten significantly associated signalling cascades, prominently featuring steroid hormone biosynthesis, ovarian steroidogenesis, prolactin signalling, endocrine resistance, metabolic pathways, and the estrogen signalling pathway (Figure 5 A & Supplementary Table 8). Notably, the presence of estrogen signalling within the top enriched pathways provides mechanistic validation for the ESR1-dominated network topology observed earlier, reinforcing the centrality of estrogen-mediated neuroendocrine regulation in age- and sex-associated cognitive decline. The apparent enrichment of cancer-related pathways reflects the well-recognized molecular overlap between estrogen signaling, cell-survival regulation, and metabolic control shared by both tumor biology and neurodegeneration, and therefore indicates network-level endocrine convergence rather than nonspecific off-target effects.

To achieve a systems-level interpretation, an integrated plant-bioactive compound-target-pathway interaction network was constructed, linking phytochemical constituents with hub-bottleneck genes and enriched KEGG pathways. This

framework illustrates extensive cross-talk between steroidogenesis, endocrine signaling, lipid metabolism, and cancer-related pathways, thereby depicting the multi-target pharmacological architecture underlying the investigated polyherbal formulation (Figure 5 B).

Further pathway mapping using Pathview highlighted hub-bottleneck genes embedded within the estrogen signaling cascade, with core regulators visually emphasized, demonstrating their direct participation in estrogen-responsive transcriptional and survival signaling (Figure 6). The convergence of network topology, GO/KEGG enrichment, and pathway mapping collectively establishes ESR1-centered estrogen modulation as the principal mechanistic axis of the formulation.

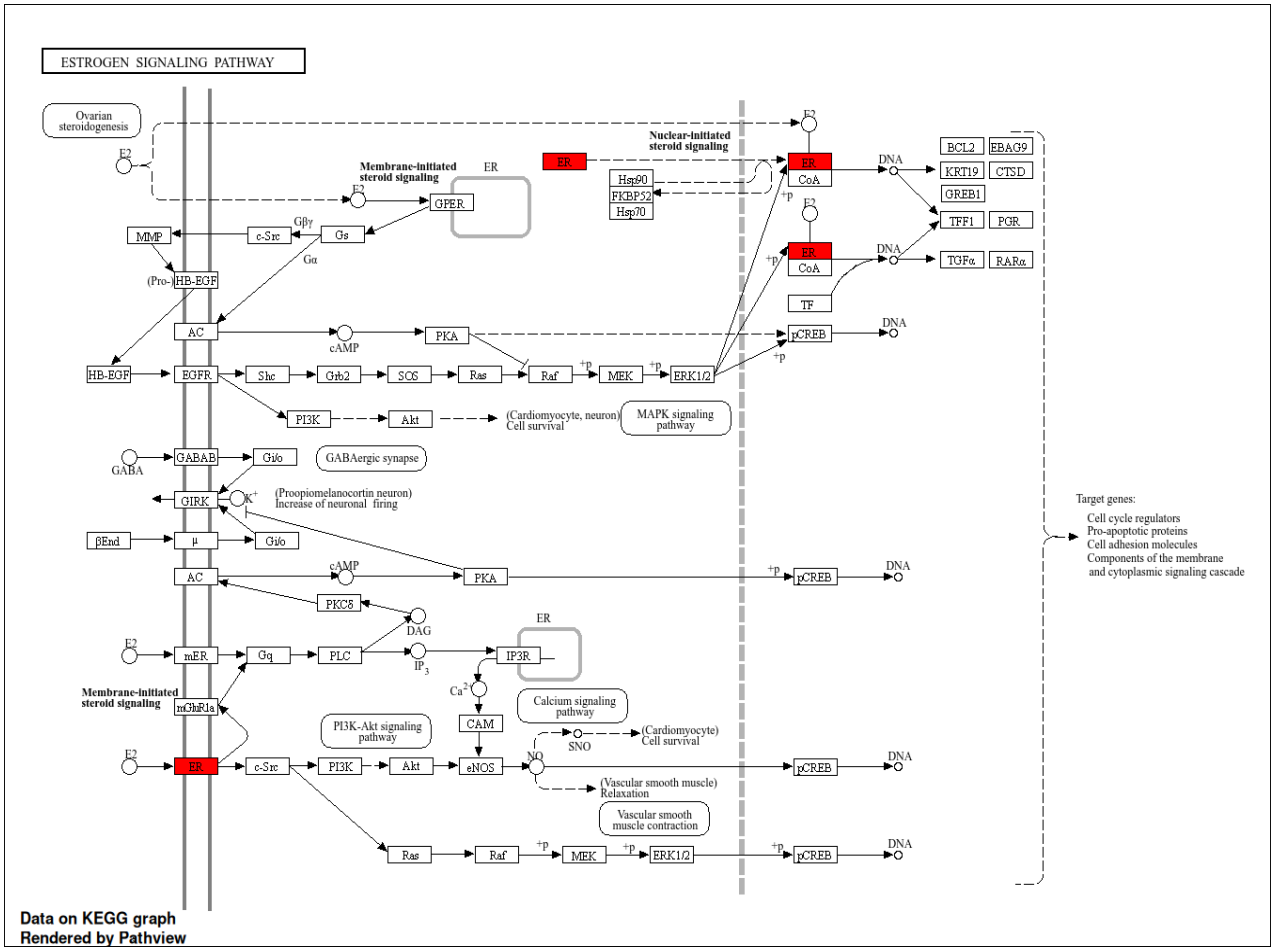

Figure 6. Estrogen signaling pathway highlighting hub-bottleneck gene involvement. Key hub-bottleneck genes are mapped onto the estrogen signaling cascade, with core regulators highlighted in red, demonstrating their direct participation in estrogen-mediated transcriptional regulation, steroidogenesis, and downstream survival signaling relevant to cognitive decline.

Importantly, this integrative evidence provides a rational foundation for downstream molecular docking, justifying the focused evaluation of the four ESR1-associated phytocompounds–β-sitosterol, stigmasterol, emodin, and diosgenin–against estrogen receptor targets. By prioritizing compounds and pathways supported simultaneously by network centrality, enrichment significance, and pathway localization, the subsequent docking analysis is positioned to deliver mechanistically coherent and biologically meaningful validation of estrogen-modulating neuroprotection.

3.6 Molecular Docking Analysis of ESR1-Phytocompound Interactions

To structurally validate the estrogen-centered mechanism predicted from network and enrichment analyses, focused molecular docking was performed between ESR1 (PDB ID: 1A52) and four prioritized phytoconstituents (β-sitosterol, stigmasterol, emodin, and diosgenin), using estradiol as the reference ligand. Binding energies and residue-level interactions are summarized in Table 3, and 2D interaction maps are shown in Figure 7A-E. Estradiol exhibited a docking score of -8.3 kcal/mol, forming hydrogen bonds with ARG(B:90) and PHE(B:100) alongside multiple stabilizing alkyl and π-anion interactions within the canonical ESR1 pocket. Among phytocompounds, emodin showed the strongest affinity (-9.3 kcal/mol), exceeding estradiol, with multiple hydrogen bonds (ARG-90, LEU-42, LEU-83) and additional π-π/alkyl contacts involving PHE(B:100), LEU(B:87), ALA(B:46), ILE(B:120), HIS(B:220), and MET(B:117), indicating robust cavity stabilization. β-Sitosterol demonstrated near-reference affinity (-8.2 kcal/mol) through hydrogen bonding with GLN(B:110) and extensive hydrophobic interactions across MET(B:84), LEU(B:80/83), ALA(B:46), PHE(B:100/121), MET(B:38/117), and VAL(B:114). Diosgenin showed moderate binding (-7.1 kcal/mol) with hydrogen bonds to ASN(B:228) and PRO(B:231) plus supporting hydrophobic contacts, while stigmasterol

displayed lower but stable affinity (-6.7 kcal/mol) with ASN(B:228)-mediated hydrogen bonding and alkyl/π-alkyl interactions. Overall, all four phytoconstituents occupied the canonical estradiol-binding region of ESR1 (Figure 7), with emodin and β-sitosterol demonstrating affinities comparable to or greater than the endogenous ligand. These results provide structural validation of ESR1 modulation, reinforcing the ESR1-centric neuroprotective mechanism inferred from systems pharmacology and pathway enrichment analyses.

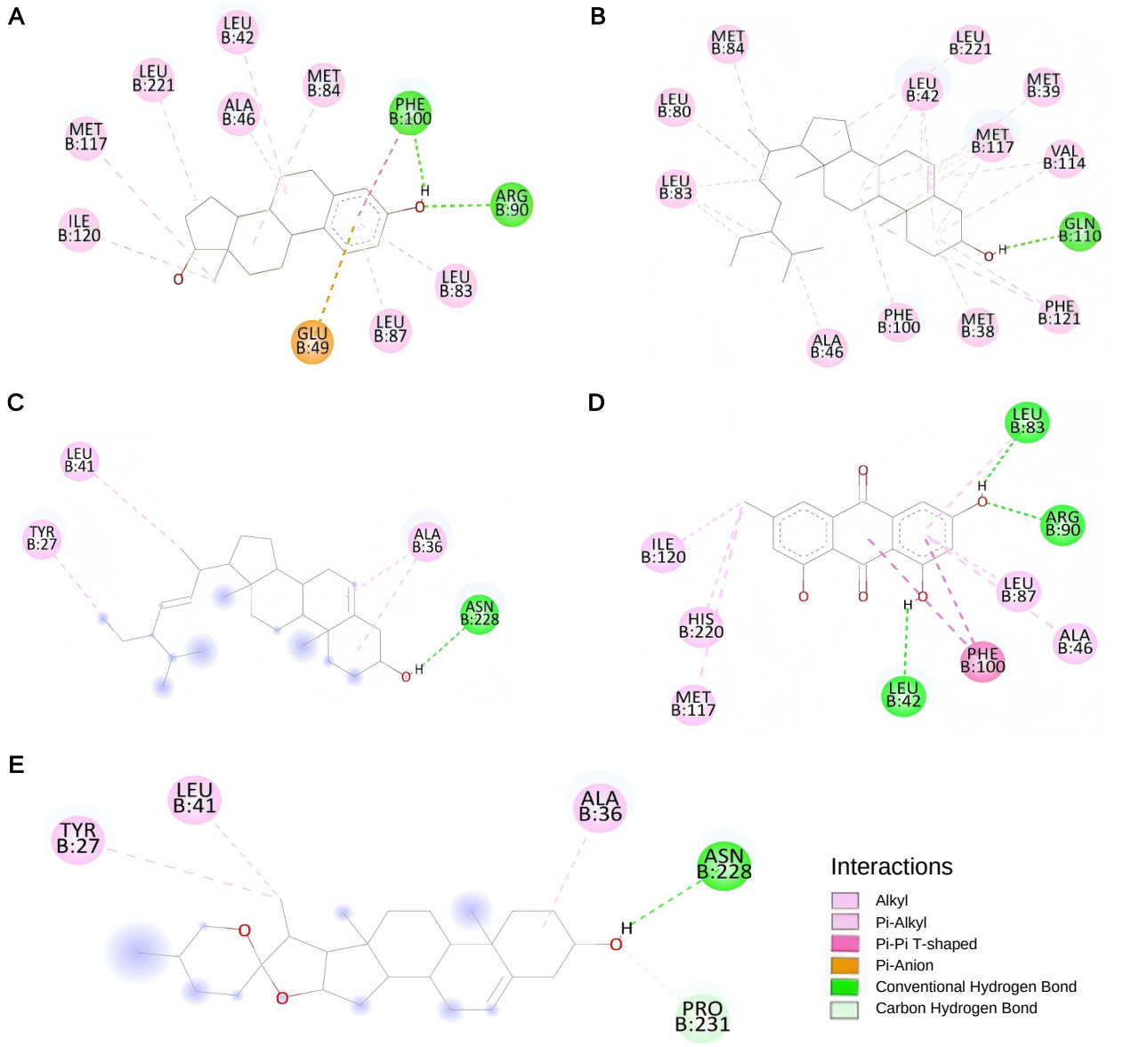

Figure 7. Two-dimensional interaction profiles of ESR1-ligand complexes. 2D binding interaction maps illustrating residue-level contacts within the ESR1 ligand-binding domain for (A) estradiol (reference), (B) β-sitosterol, (C) stigmasterol, (D) emodin, and (E) diosgenin. Hydrogen bonds and hydrophobic/π-mediated interactions demonstrate consistent engagement of the canonical estradiol-binding cavity, with emodin and β-sitosterol showing strong stabilizing interactions, supporting ESR1-centered estrogen-modulating activity.

Table 3. Molecular docking interactions of selected phytoconstituents with ESR1 (PDB ID: 1A52).

Phytoconstituent Name (PubChem ID)	ESR1 (1A52)	Hydrogen bond interactions	Other interactions
Estradiol (Reference)	-8.3	ARG (B:90) PHE (B:100)	GLU (B:49)–π-anion interaction with aromatic ring of ligand; LEU (B:42)–Alkyl interaction ALA (B:46)–Alkyl interaction MET (B:84)–Alkyl interaction MET (B:117)__Alkyl interaction ILE (B:120)–Alkyl interaction LEU (B:221)–Alkyl interaction LEU (B:83)–Alkyl interaction
Beta-sitosterol (222284)	-8.2	GLN (B:110) Å	MET (B:84)–Alkyl interaction LEU (B:80)–Alkyl interaction LEU (B:83)–Alkyl interaction ALA (B:46)–Alkyl interaction PHE (B:100)–π-alkyl interaction MET (B:38)–Alkyl interaction PHE (B:121)–π-alkyl interaction VAL (B:114)–Alkyl interaction MET (B:117)–Alkyl interaction
Stigmasterol (5280794)	-6.7	ASN (B:228)	LEU (B:41)–Alkyl interaction TYR (B:27)–π-alkyl interaction ALA (B:36)–Alkyl interaction
Emodin (3220)	-9.3	Arg-90:1.8 Å; Leu-42:2.5 Å; Leu-42:3.3 Å; Leu-83:2.3 Å	PHE (B:100)–π-π T-shaped interaction with aromatic ring LEU (B:87)–Alkyl interaction ALA (B:46)–Alkyl interaction ILE (B:120)–Alkyl interaction HIS (B:220)–Alkyl interaction MET (B:117)–Alkyl interaction
Diosgenin (99474)	-7.1	ASN (B:228) PRO (B:231)	TYR (B:27)–π-alkyl interaction LEU (B:41)–Alkyl interaction ALA (B:36)–Alkyl interaction

3.7 Molecular Dynamics Stability of ESR1-Phytocompound Complexes

To further validate docking-derived interactions, 25-ns molecular dynamics simulations were performed for ESR1 complexes with the reference ligand estradiol and the selected phytocompounds. RMSD trajectory analysis demonstrated that emodin and β-sitosterol exhibited brief initial fluctuations followed by rapid stabilization below ~3 Å, closely paralleling the stability profile of estradiol, thereby indicating comparable binding robustness within the ESR1 ligand-binding domain. Notably, the emodin-ESR1 complex maintained sustained stability after ~16 ns, suggesting particularly strong conformational accommodation. Other ligand-bound complexes also remained within an acceptable fluctuation range throughout the simulation window, supporting overall structural integrity of ESR1-ligand interactions (Figure 8A).

Consistent with RMSD findings, RMSF residue-level fluctuation analysis revealed minimal internal flexibility across ESR1, with deviations largely confined to terminal loop regions rather than the core ligand-binding pocket, indicating stable ligand engagement and preserved receptor conformation (Figure 8B). Collectively, molecular dynamics simulations confirm that key phytoconstituents–especially emodin and β-sitosterol–maintain ESR1 structural stability comparable to the endogenous ligand estradiol. These results provide dynamic validation of estrogen-receptor modulation, reinforcing the ESR1-centered neuroprotective mechanism inferred from network pharmacology, enrichment analysis, and molecular docking.

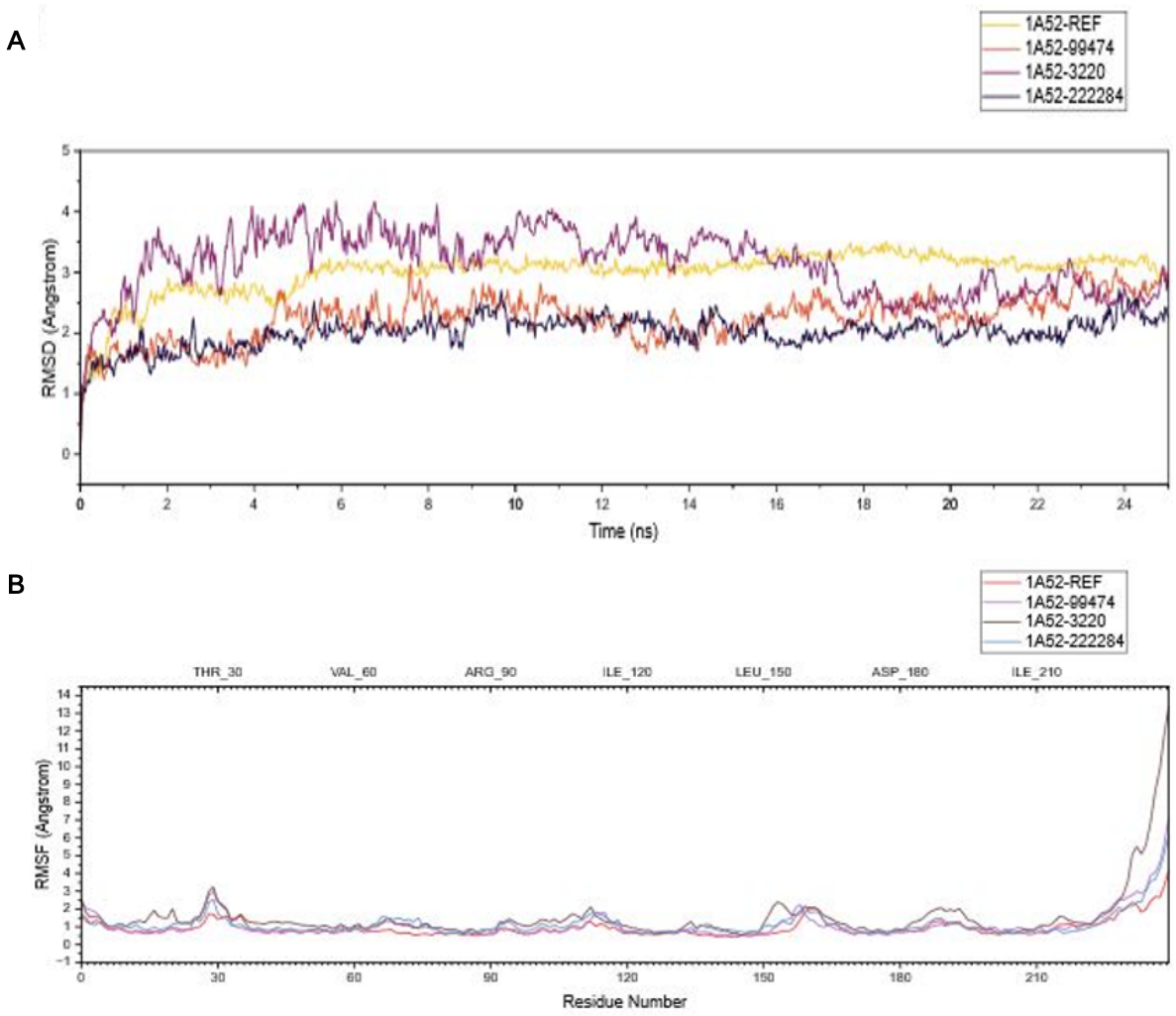

Figure 8. Molecular dynamics stability of ESR1-ligand complexes. (A) RMSD trajectories over 25 ns showing rapid stabilization of emodin and β-sitosterol below ~3 Å, comparable to estradiol, indicating stable binding. (B) RMSF residue fluctuation profile demonstrating minimal internal flexibility with fluctuations restricted to terminal regions, confirming preservation of the ESR1 ligand-binding architecture.

4. Discussions

AD remains a multifactorial neurodegenerative disorder for which currently available therapies provide only symptomatic relief without effectively modifying disease progression. A critical but often under-integrated dimension of AD pathology is its pronounced sex disparity, with women exhibiting higher prevalence, faster decline, and stronger association with post-menopausal estrogen loss. In this context, therapeutic strategies capable of restoring estrogen-mediated neuroprotection without systemic hormone replacement represent an important unmet need. The present systems pharmacology-driven investigation was therefore designed to evaluate whether a polyherbal phytoformulation could mechanistically converge on estrogen-centered regulatory networks relevant to cognitive decline.

Network pharmacology analysis first revealed that ESR1 occupies the dominant hub-bottleneck position within the PPI landscape, supported by the highest degree and betweenness centrality among all prioritized genes. This topological prominence indicates that ESR1 functions not merely as an associated receptor but as a systems-level regulatory core integrating steroidogenesis, lipid metabolism, endocrine signaling, and transcriptional control. The concurrent presence of HMGCR, CYP19A1, CYP17A1, ESR2, AR, SHBG, and SREBF2 further emphasizes a tightly interconnected cholesterol-steroid-estrogen regulatory axis, which is highly relevant to both brain aging and neurodegenerative vulnerability [39]. Such convergence supports growing evidence that disrupted estrogen signaling and metabolic dysfunction are mechanistically linked drivers of AD progression, particularly in females [40].

Functional enrichment analyses provided additional biological validation for this ESR1-centered framework. GO results consistently highlighted intracellular estrogen receptor signaling, steroid biosynthesis and metabolism, androgen processing, estradiol responsiveness, and sex differentiation, while cellular localization pointed toward endoplasmic reticulum-associated steroidogenic compartments and transcriptional chromatin machinery. Molecular function enrichment further confirmed nuclear hormone receptor activity and estrogen response element binding as dominant

regulatory themes. Complementary KEGG pathway analysis reinforced these findings by identifying estrogen signaling, ovarian steroidogenesis, prolactin signaling, endocrine resistance, and steroid hormone biosynthesis among the most significantly enriched pathways. Together, these multilayered enrichment results strongly indicate that the investigated phytochemical network converges on endocrine and estrogen-dependent neuroregulatory biology, rather than unrelated inflammatory or purely amyloid-centric mechanisms.

The integrated plant-bioactive-target-pathway network further demonstrated coordinated cross-talk between steroidogenic, metabolic, and cancer-related endocrine pathways, reflecting the inherent multi-target pharmacology of polyherbal systems. Importantly, mapping of hub-bottleneck genes onto the estrogen signaling cascade confirmed their direct participation in receptor-mediated transcriptional and survival signaling, thereby providing mechanistic continuity between network topology and biological function. This convergence created a strong rationale for structure-based validation focused specifically on ESR1, rather than broad multi-target docking lacking systems justification. The concurrent enrichment of cancer-associated pathways within this network should therefore be interpreted within the context of shared endocrine, metabolic, and survival signaling architecture rather than unintended oncogenic targeting. Many pathways annotated under cancer biology—particularly those involving estrogen signaling, PI3K-AKT/MAPK cascades, and transcriptional growth regulation—represent fundamental regulators of cellular stress adaptation, mitochondrial integrity, and controlled survival, processes equally critical to neuronal maintenance in neurodegeneration [41,42]. In polyherbal systems, such pathway overlap reflects network-level polypharmacology and homeostatic modulation, consistent with an adjunctive, systems-restorative therapeutic role rather than pathway-specific cytotoxic intervention.

Molecular docking analysis substantiated this prediction by demonstrating that all four prioritized phytoconstituents occupy the canonical estradiol-binding pocket of ESR1 with energetically favorable interactions. Notably, emodin displayed stronger binding affinity than estradiol, while β-sitosterol showed near-equivalent stability, indicating genuine potential for functional estrogen receptor modulation rather than nonspecific hydrophobic association. The interaction profiles—comprising hydrogen bonding, π-stacking, and hydrophobic stabilization within key ligand-binding residues—mirror canonical estrogenic engagement, suggesting that these phytochemicals may act as selective estrogen-modulating scaffolds.

Dynamic validation through 25-ns molecular dynamics simulations further reinforced these structural findings. Both emodin-ESR1 and β-sitosterol-ESR1 complexes demonstrated rapid RMSD stabilization below ~3 Å, closely matching the endogenous ligand and indicating stable conformational accommodation within the receptor cavity. Minimal RMSF fluctuations confined to terminal regions rather than the ligand-binding core confirm preservation of receptor structural integrity during ligand engagement. Collectively, docking and molecular dynamics results provide complementary static and dynamic evidence supporting stable ESR1 modulation by phytoconstituents.

From a therapeutic perspective, these findings align with the emerging concept that phytoestrogen-like multi-target agents may offer safer long-term neuroprotection compared with systemic HRT, which is limited by cardiovascular and oncogenic risks [43]. By simultaneously influencing steroidogenesis, lipid metabolism, and receptor-mediated transcription, polyherbal formulations may restore network-level endocrine balance rather than delivering supraphysiological hormone exposure. This systems-restorative paradigm is particularly relevant to female cognitive aging, where gradual estrogen decline intersects with metabolic and inflammatory vulnerability [44].

Nevertheless, several limitations should be acknowledged. The present study is computational and predictive, lacking *in vitro* receptor activation assays, transcriptomic validation, or *in vivo* neurobehavioral confirmation. Simulation timescales were also relatively short compared with biological processes, and polyherbal pharmacokinetics in the human brain remain uncertain. Future studies integrating cellular estrogen signaling assays, BBB transport evaluation, and animal cognition models will be essential to translate these findings toward clinical relevance.

Despite these limitations, the current integrative framework provides coherent multiscale evidence—from network topology to pathway biology to structural dynamics—supporting ESR1-centered estrogen modulation as the principal mechanism through which the investigated polyherbal formulation may counter age- and sex-associated cognitive decline. This work therefore establishes a mechanistically grounded foundation for phytoestrogen-based neuroprotective strategies and highlights the value of systems pharmacology in decoding complex herbal therapeutics for AD.

5. Conclusion

This study establishes a coherent estrogen-centered mechanistic framework through which a rationally designed polyherbal phytoformulation may counter age- and sex-associated cognitive decline. Convergent evidence across network topology, functional enrichment, structural binding, and dynamic stability consistently identified ESR1-mediated signaling as the dominant regulatory axis linking steroidogenesis, metabolic balance, and neuroprotective transcriptional control. Among the screened phytoconstituents, emodin and β-sitosterol demonstrated particularly strong potential to stably engage the ESR1 ligand-binding domain, supporting their role as putative phytoestrogenic modulators capable of restoring endocrine-dependent neuronal resilience. These findings collectively highlight the therapeutic relevance of multi-target, systems-restorative phytomedicine as a complementary strategy to address the complex and sex-biased biology of AD. While experimental validation remains necessary, the present integrative

analysis provides a mechanistically grounded foundation for advancing ESR1-focused phytoestrogenic interventions toward translational neuroprotection and rational development of safer, endocrine-modulating therapeutics for cognitive aging.

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Data Availability Statement

All data and materials used to support the findings of this study are included within the article.

Conflict of Interest

The authors declare no conflict of interest.

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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